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CD4+ T cells enhance the unloaded shortening velocity of airway smooth muscle by altering the contractile protein expression

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Key points

- Activated CD4+ T cells enhance the contractility of airway smooth muscle.
- In order to enhance contractility, contact between CD4+ T cells and smooth muscle is required.
- The enhanced contractility is correlated with increased levels of fast myosin isoform.
- Our data suggest that inflammatory cells promote airway smooth muscle hypercontractility in airway hyper-responsiveness and asthma.

Abstract Abundant data indicate that pathogenesis in allergic airways disease is orchestrated by an aberrant T-helper 2 (Th2) inflammatory response. CD4+ T cells have been localized to airway smooth muscle (ASM) in both human asthmatics and in rodent models of allergic airways disease, where they have been implicated in proliferative responses of ASM. Whether CD4+ T cells also alter ASM contractility has not been addressed. We established an *in vitro* system to assess the ability of antigen-stimulated CD4+ T cells to modify contractile responses of the Brown Norway rat trachealis muscle. Our data demonstrated that the unloaded velocity of shortening (V_{max}) of ASM was significantly increased upon 24 h co-incubation with antigen-stimulated CD4+ T cells, while stress did not change. Enhanced V_{max} was dependent upon contact between the CD4+ T cells and the ASM and correlated with increased levels of the fast (+)insert smooth muscle myosin heavy chain isoform. The levels of myosin light chain kinase and myosin light chain phosphorylation were also increased within the muscle. The alterations in mechanics and in the levels of contractile proteins were transient, both declining to control levels after 48 h of co-incubation. More permanent alterations in muscle phenotype might be attainable when several inflammatory cells and mediators interact together or after repeated antigenic challenges. Further studies will await new tissue culture methodologies that preserve the muscle properties over longer periods of time. In conclusion, our data suggest that inflammatory cells promote ASM hypercontractility in airway hyper-responsiveness and asthma.

Introduction

Asthma is an inflammatory disease of the airways characterized in part by airway hyper-responsiveness (AHR), an exaggerated response of airway smooth muscle (ASM) to contractile stimuli (Bateman *et al.* 2008). The contribution of T-helper 2 (Th2)-biased airway inflammation to pathogenesis in asthma has been the focus of abundant research and still receives a lot of attention (Lloyd & Hessel, 2010), whereas the role of ASM remains elusive (Ozier *et al.* 2011). It is generally agreed, however, that airway narrowing mediated by ASM contraction is the culminating point in asthma (Brusasco & Pellegrino, 2003; Bossé, 2012; Chin *et al.* 2012).

Chronic airway inflammation is thought to contribute to most clinical manifestations of AHR in asthma (Berend *et al.* 2008), presumably by enhancing ASM contractility. Supporting this notion is the fact that mast cells, T cells, eosinophils and macrophages are found within the ASM layers of human asthmatic subjects (Brightling *et al.* 2002; Begueret *et al.* 2007; Ramos-Barbon *et al.* 2010). Airway hyper-responsiveness was initially reported to be proportional to the degree of mast cell infiltration within the ASM layers (Brightling *et al.* 2002), but more recently, among inflammatory cells, T cells were the only cells found to increase significantly in the smooth muscle of asthmatic subjects (Begueret *et al.* 2007). The number of activated $CD4^+$ T cells in the airway wall of mice with experimental asthma is also associated with AHR (Zosky *et al.* 2009). Moreover, asthmatic ASM cells express ligands that interact with and activate associated T cells (Lazaar *et al.* 1994; Burgess *et al.* 2005), supporting the notion that ASM also displays pro-inflammatory and immunomodulatory functions (Ozier *et al.* 2011).

Adoptively transferred T cells have also been found in apparent contact with ASM in Brown Norway (BN) rats with experimental asthma (Ramos-Barbon *et al.* 2005). Significantly, $CD4^+$ T cells from sensitized BN rats adoptively transferred to naive rats induced an increase in ASM mass after antigen challenge, demonstrating an *in vivo* modulation of ASM function by $CD4^+$ T cells (Ramos-Barbon *et al.* 2005). This interaction resulted in cross-activation and increased survival of both cell types (Ramos-Barbon *et al.* 2005) and is consistent with previous data from human cells, where T cell activation by human ASM cells increased the release of interleukin-13 (Veler *et al.* 2007) as well as other Th2 cytokines (Lloyd & Hessel, 2010). Together, these data provide evidence that smooth muscle cells can promote survival, activation and/or cytokine production from target T cells. However, the possibility that $CD4^+$ T cells alter contractile protein expression and/or ASM function, either directly or via cytokine release, has not been assessed.

The aim of this study was to determine whether exposure of ASM cells to total splenocytes or purified $CD4^+$ T cells, activated *in vitro* with antigen, altered contractile protein expression and ASM function. Thus, we investigated the mechanical properties and contractile protein expression of BN rat trachealis after incubation with ovalbumin (OVA)-stimulated total splenocytes or OVA-stimulated $CD4^+$ T cells.

Methods

All procedures were approved by the McGill University Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

Harvesting and culture of splenocytes and isolation of $CD4^+$ T cells

Male BN rats (ssNOlaHsd, Harlan UK Ltd, Shardlow, Derbyshire, England; 8–10 weeks old) were injected I.P. with 200 μg ml^{-1} OVA (grade V; Sigma-Aldrich, Oakville, Ontario, Canada) in Imject alum (Thermo-Scientific, Ottawa, Ontario, Canada). Two weeks later, the rats were killed by I.P. injection of 150 mg kg^{-1} of sodium pentobarbital. Spleens were harvested and placed in 50 ml centrifuge tubes containing sterile phosphate buffer solution (PBS). A single-cell suspension of total splenocytes was prepared after lysis of red blood cells with ammonium chloride–potassium buffer and cultured with OVA to induce re-activation and proliferation of the OVA-specific T cells generated *in vivo* by I.P. OVA administration. Cells were cultured at a concentration of 5×10^6 cells ml^{-1} for 4 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units ml^{-1} interleukin-2, 100 μg ml^{-1} OVA and 1% penicillin–streptomycin. After 4 days of culture, the splenocytes were either harvested for co-culture with the ASM directly or further processed using EasySep negative selection kit (Stemcell Technologies, Vancouver, British Columbia, Canada) to purify $CD4^+$ T cells. The purity of the $CD4^+$ T cells was assessed by flow cytometry using a CD4 APC antibody (BD Pharmingen, San Diego, CA, USA) and was reliably >95% (Fig. 1*A*), consistent with images obtained using both bright field (Fig. 1*B*) and immunofluorescence microscopy using a CD4 Alexa488 antibody (Biolegend, San Diego, CA, USA; Fig. 1*C*).

Harvesting of tracheae and incubation with inflammatory cells

Two weeks after an I.P. OVA injection, the BN rats were killed and the tracheae dissected and placed in calcium-free Krebs–Henseleit (KH) solution [composition (mM): 118 NaCl, 4.5 KCl, 2.5 $MgSO_4$, 1.2 KH_2PO_4, 25.5 $NaHCO_3$ and 10.0 glucose, pH 7.4; aerated with 95% O_2–5% CO_2

for at least 30 min]. Loose connective tissue was then removed and the tracheae were cut transversely into rings, each containing a single ring of cartilage and a strip of smooth muscle from the dorsal side of the trachea. The most caudal ring and the cranial third of each trachea were discarded, so that up to 10 rings were obtained from each trachea. The inner surface of each ring was gently rubbed with a small metal pin or cotton swabs to remove the epithelium.

Given that the ASM properties are known to vary along the length of the trachea (Florio *et al.* 1996), we standardized the sites from which the rings were obtained (lower part of trachea for the 24 h data points and middle part of trachea for the 48 h data points). Furthermore, adjacent rings were paired as test and control samples to minimize differences due to anatomical origin. After 24 or 48 h of incubation, the rings were either used for mechanics measurements or were snap-frozen in liquid N_2 and stored at $-80°C$ for Western blot analysis.

The rings were placed in 24-well cell culture plates (Costar; Corning, Tewksbury, MA, USA) that contained either splenocytes (5×10^6 cells ml^{-1}) or CD4^{+} T cells (5×10^6 cells ml^{-1}) in complete DMEM containing 0.5% FBS and 100 units ml^{-1} interleukin-2 + 1% penicillin–streptomycin (test) or in complete DMEM without cells (control) and incubated for 24 or 48 h at 37°C in humidified air containing 5% CO_2. The cells in each well were lightly mixed by pipetting up and down several times before adding the tracheal rings. The concentration of cells used was based on the work of Ramos-Barbon *et al.* (2005), i.e. the total number of lung lymphocytes in naive BN rats adoptively transferred with CD4^{+} T cells and challenged with OVA ranged from 19×10^6 to 30×10^6 cells (see Fig. 1G of Ramos-Barbon *et al.* 2005).

Muscle to T-cell contact assay

In some experiments, 12-well Transwell plates (Corning Inc., New York, NY, USA) were used to test whether or not direct contact between ASM cells and T cells is important for the observed responses. The tracheal rings were placed in the bottom chamber in DMEM, while CD4^{+} T cells were placed in the upper chamber. The T cells were separated from the ASM by a membrane that had 0.4 μm pores, which would allow cytokines released from the CD4^{+} T cells to reach the ASM cells but prevent direct contact between ASM and T cells. The following three experimental conditions were tested: (1) control ASM incubated in medium alone; (2) ASM co-cultured in direct contact with CD4^{+} T cells; and (3) ASM co-cultured without direct contact with CD4^{+} T cells. After 24 h of incubation at 37°C in humidified air containing 5% CO_2, the contractile properties of the tracheal rings were assessed.

Muscle strip mechanics measurements

The control and test rings from a matched pair were tested on the same day and in random order. Immediately before testing, the ring was taken from the culture medium and placed in oxygenated calcium-free KH solution for 15–30 min and then the cartilage was cut on both sides of the muscle to yield a muscle strip with $\sim$1–2 mm of cartilage at each end. T-shaped aluminum foil clips (Ford *et al.* 1977) were wrapped around the cartilage segments to attach the muscle horizontally in the experimental chamber (Bullimore *et al.* 2011) at one end to the length controller (model 322C-I; Aurora Scientific Inc., Aurora, Ontario, Canada) and at the other end to the force transducer (model 400A; Aurora Scientific Inc.). The muscle length and force were computer controlled (software# 600A; Aurora Scientific Inc.).

The muscle strips were mounted in calcium-free KH solution to suppress basal tone. Length was set to a reference length (L_{ref}) defined as 1.25 × unstretched length. Defining L_{ref} in this way gave more reproducible results in these small tissue strips than using the *in situ*
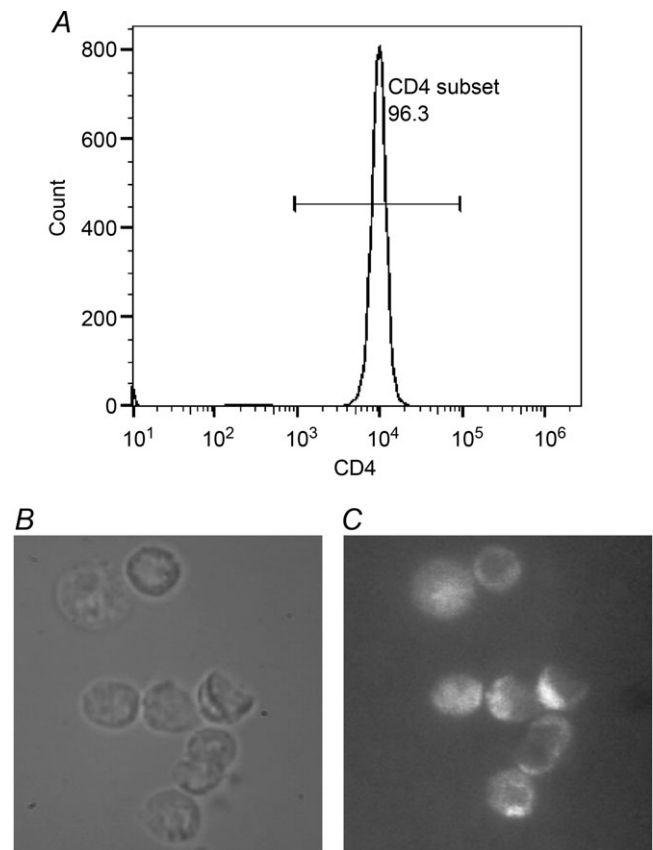

Figure 1. CD4^{+} T cell purity
A, flow cytometric quantification of the purity of the CD4^{+} T cells (>95%) after magnetic selection. CD4^{+} T cells viewed by bright field imaging (*B*) and by fluorescence microscopy after treatment with CD4 Alexa488 antibody (*C*).

length (Bullimore *et al.* 2011). Muscle strip length and width at the L_{ref} were measured using a microscope eyepiece reticule or using a Hitachi Camera (KP-D20A/B) with ImageJ software imagej.nih.gov/ij/(IJ 1.46r; NIH, USA). After setting the muscle strip at the L_{ref}, the KH solution was changed for one containing Ca^{2+} (2.5 mM $CaCl_2$). The solution was stored in a reservoir, where it was aerated with 95% O_2–5% CO_2 and circulated through the experimental chamber at ~3 ml min^{-1}, except for the periods of methacholine (MCh) stimulation. The chamber temperature was maintained at ~37°C by a water jacket.

The muscle strips were then equilibrated by stimulating every 10 min for at least 1 h with a KH solution containing KCl (final concentration 80 mM KCl), followed by two periods of supramaximal stimulation (2 min) with MCh (10^{-4} M, acetyl-β-methylcholine chloride; Sigma Chemical Co., Oakville, Ontario, Canada) separated by washout periods of 15–20 min. The force–velocity measurements were made during a third period of supramaximal MCh stimulation, starting the quick releases 2–5 min after the beginning of activation. Briefly, the shortening velocity at a given force was measured by clamping the force at that level and allowing the muscle strip to shorten for 110 ms. The strip was then stretched back to the L_{ref}, and force was allowed to stabilize before the next force clamp. Force clamps to levels ranging between 5 and 80% isometric force were performed in random order to construct the force–velocity curve.

Phosphorylation analysis of regulatory light chain of smooth muscle myosin (LC$_{20}$)

To verify whether or not the level of LC$_{20}$ phosphorylation was altered by the presence of the CD4$^+$ T cells, muscle strips (control and test) were mounted at the L_{ref} in a vertical experimental set-up with a 10 g force transducer (World Precision Instruments, Sarasota, FL, USA), and equilibrated for 1 h in KH solution with Ca^{2+}. The muscle strips were then contracted for 5 min with 10^{-5} M MCh, and then rapidly frozen using liquid nitrogen-prechilled clamps. The strips were immersed for 2 min in dry ice-cooled acetone containing 10% trichloroacetic acid (w/v) and 20 mM dithiothreitol (Sigma Chemical Co.), washed three times in acetone containing 10 mM dithiothreitol and then dried for 30 min at room temperature.

Western blot analysis

The contractile protein expression in the BN rat trachealis muscle exposed to the total splenocytes, CD4$^+$ T cells or medium was examined by Western blot analysis. After 24 or 48 h of co-culture, the tracheal rings were collected, snap-frozen in liquid nitrogen and kept at −80°C until assayed. Proteins were extracted as described by Gil *et al.* (2006). Equal quantities,

determined by a standard Bradford assay, were resolved on 4–20% SDS-PAGE gradient gels, after which the proteins were electroblotted onto Polyvinylidene flouride (PVDF) membranes (Bio-Rad, Mississauga, Ontario, Canada). The membranes were cut below the 75 kDa molecular marker and sections blocked at room temperature with either 5% non-fat dry milk or 2% BSA in Tris-buffered saline. The upper membranes were probed with the monoclonal antibody that recognizes myosin light chain kinase (MLCK; 1:500 dilution; Sigma-Aldrich) and the polyclonal antibody that recognizes all smooth muscle myosin heavy chain (SMMHC) isoforms (1:2000 dilution; Biomedical Technologies Inc., Stoughton, MA, USA) or the polyclonal antibody that specifically recognizes the (+)insert SMMHC isoform (1:200 dilution) and that was raised against the peptide sequence QGPSFAY (Antibody Resource for Neuroscience Research Core at the Montreal Neurological Institute, Canada). The bottom portions of the membranes were probed by the monoclonal mouse antibody that recognizes glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000 dilution; Ambion, Burlington, Ontario, Canada), which was used as a loading control, and the monoclonal mouse antibody that recognizes smooth muscle α-actin (1:5000 dilution; Sigma-Aldrich). For detection, secondary antibodies were coupled with horseradish peroxidase (1:10,000 dilution; GE Healthcare, Baie-d'Urfé, Quebec, Canada) except for detection of the (+)insert SMMHC isoform, for which amplification was performed by using biotinylated secondary antibody (1:10,000 dilution; Dakocytomation, Burlington, Ontario, Canada) and streptavidin coupled to horseradish peroxidase (1:40,000 dilution; Thermo Fisher Scientific, Whitby, Ontario, Canada).

To assess the myosin LC$_{20}$ phosphorylation level, the protein extraction and Phos-tag^{TM} (Wako chemicals, Amagasaki, Japan) SDS-PAGE were performed according to a published protocol (Takeya *et al.* 2008) with minor modifications. Briefly, dry muscle strips were cut into small pieces and homogenized in SDS-gel sample buffer (4% SDS, 10% glycerol, 100 mM dithiothreitol and 65 mM Tris–HCl, pH 6.8) and 0.04% (w/v) Bromophenol Blue. The samples were incubated for 2 h at 26°C with continuous mixing at 400 r.p.m., sonicated for 3 min and heated at 98°C for 5 min. The proteins were resolved by Phos-tag^{TM} gel electrophoresis and then transferred to PVDF membranes overnight (45 V) at 4°C. The membranes were fixed with 0.5% glutaraldehyde (Fisher Scientific) for 45 min, blocked with 0.3% I-Block (Tropix, New York, NY, USA) for 2 h at room temperature and the LC$_{20}$ visualized by probing the membrane with polyclonal rabbit anti-LC$_{20}$ antibody (Santa Cruz Biotechnology, Dallas, TX, USA). The LC$_{20}$ phosphorylation is reported as the ratio of phosphorylated LC$_{20}$ to total LC$_{20}$.

Proteins were detected with enhanced chemiluminescence substrate (SuperSignal West Dura; Thermo

Fisher Scientific) for all proteins except LC_{20}, which was detected using Luminata Forte (Millipore, Billerica, MA, USA). Quantification was performed with a Fluorchem 8500 imaging system using AlphaEase software (Alpha Innotech, Santa Clara, CA, USA). Western blots from each independent experiment were performed in triplicate.

Data analysis

The muscle strip cross-sectional area was determined by multiplying the measured width by a thickness of 0.1 mm (Bullimore *et al.* 2011). Maximal stress-generating capacity was calculated by dividing the maximal force (F_{max}) by cross-sectional area of ASM and reported in kPa. The velocity of shortening during the force-clamp experiments was determined by fitting a regression line to the relationship of length to time between 60 and 110 ms after the start of the clamp, and normalizing the slope of the line to L_{ref} (length per second). The force during each force clamp was normalized to the isometric force immediately before the clamp. Hill's hyperbolic force–velocity equation (Hill, 1949) was fitted to these normalized forces and velocity data using a non-linear least-squares algorithm in Matlab (The Mathworks Inc., Natick, MA, USA). The intercept of the fitted curve with the velocity axis was taken to be the maximal shortening velocity (V_{max}). Results of stress, V_{max}, protein expression and phosphorylation levels are reported as means ± SEM, and '*n*' refers to the number of animals. All data were analysed by Student's paired *t* test except for the contact assay, which was analysed by a one-way ANOVA with Bonferroni's multiple comparison test. Significance was considered for values of $P < 0.05$ and is shown in figures by asterisks.

Results

Muscle mechanics after co-culture of ASM with OVA-stimulated splenocytes

To assess whether or not antigen-stimulated inflammatory cells altered ASM mechanical properties, we co-cultured tracheal rings with and without OVA-stimulated splenocytes and measured the muscle strip V_{max} and stress. No significant difference was found in V_{max} between control and OVA-stimulated splenocytes at 24 h (0.22 ± 0.02 l *vs.* 0.24 ± 0.02 lengths s^{-1}, respectively, $P = 0.26$) or 48 h (0.21 ± 0.04 l *vs.* 0.25 ± 0.03 lengths s^{-1}, respectively, $P = 0.23$; Fig. 2*A*). Likewise, no differences were observed for muscle stress at 24 h (83.4 ± 5.6 *vs.* 99.4 ± 8.2 kPa, $P = 0.095$; control *vs.* splenocytes) or 48 h (70.0 ± 9.27 *vs.* 69.4 ± 10.1 kPa, $P = 0.91$; control *vs.* splenocytes; Fig. 2*B*). Representative data from a control muscle strip and from one incubated with OVA-stimulated splenocytes are presented in Fig. 2*C*.
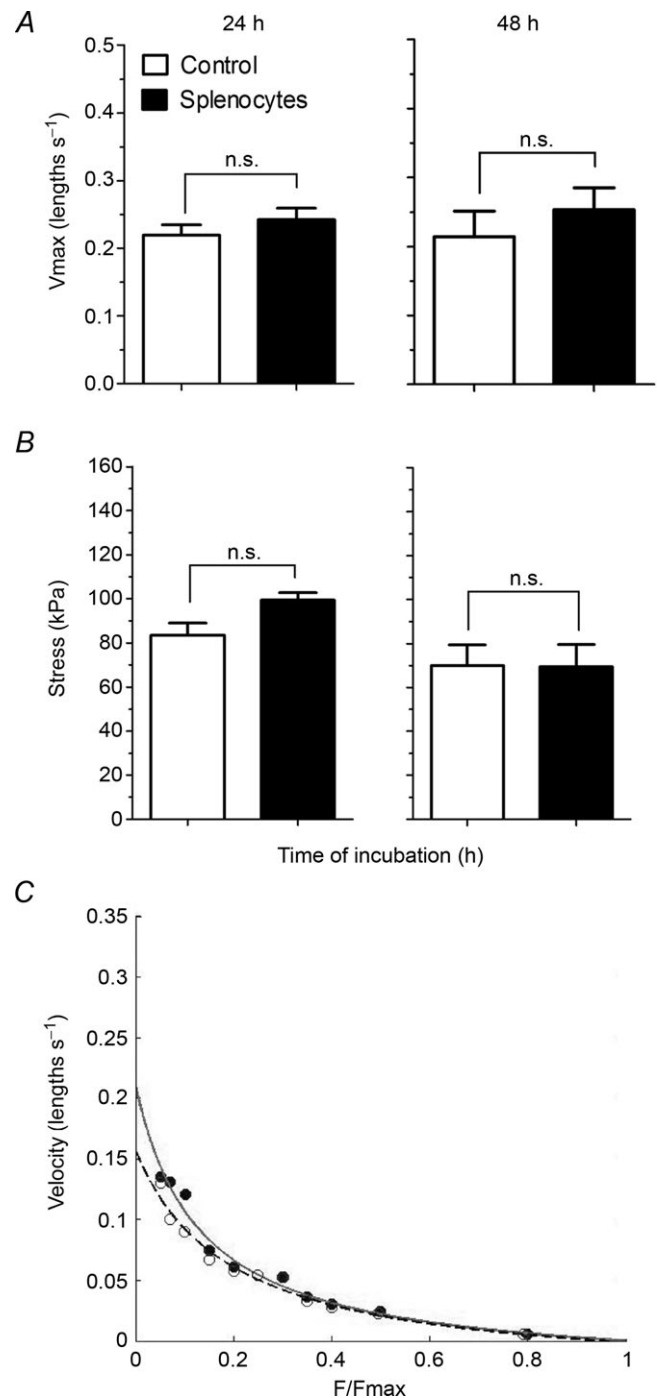

Figure 2. Mechanical properties of airway smooth muscle (ASM) incubated with ovalbumin (OVA)-stimulated splenocytes
A, unloaded velocity of shortening (V_{max}) of ASM co-cultured in the absence (open columns) or in the presence of total splenocytes (filled columns) for 24 or 48 h ($n = 5$ rats per group). *B*, stress generated by ASM incubated in the absence (open squares) or in the presence of total splenocytes (filled columns) for 24 or 48 h ($n = 6$ rats per group). *C*, representative force–velocity curves of ASM co-cultured in the absence (open circles) or in the presence of total splenocytes (filled circles). F/Fmax: force normalized to the maximal force. The force–velocity relationships were accurately fitted by the Hill hyperbolic model, $r^2 > 0.96$.

Western blot analysis of contractile proteins in ASM co-cultured with OVA-stimulated splenocytes

Contractile protein expression of ASM co-cultured with OVA-stimulated splenocytes for 24 h was also assessed. The level of MLCK was significantly increased in the splenocyte-exposed muscle strips compared with control muscle (0.19 ± 0.02 *vs.* 0.29 ± 0.03 a.u.; control *vs.* splenocytes, $P = 0.034$; Fig. 3*A*). There was no difference observed in the expression of the fast (+)insert SMMHC isoform (0.36 ± 0.03 *vs.* 0.36 ± 0.02 a.u., $P = 0.84$), total SMMHC (0.52 ± 0.03 *vs.* 0.51 ± 0.03 a.u., $P = 0.96$), α-smooth muscle actin (1.34 ± 0.03 *vs.* 1.36 ± 0.13 a.u., $P = 0.92$) or in the fast (+)insert SMMHC isoform to total SMMHC ratio (0.69 ± 0.03 *vs.* 0.70 ± 0.1, $P = 0.89$; Fig. 3*B–E*).

Mechanics of ASM after co-culture with OVA-stimulated CD4^{+} T cells

To evaluate more directly whether or not CD4^{+} T cells altered ASM mechanical properties, tracheal rings were co-cultured with or without OVA-stimulated CD4^{+} T cells, and the muscle strip V_{max} and stress were then measured. In contrast to total splenocytes, after 24 h incubation with OVA-stimulated CD4^{+} T cells, the ASM contractile function was significantly increased in V_{max} (0.15 ± 0.01 *vs.* 0.29 ± 0.02 lengths s^{-1}; control *vs.* T cells, $P = 0.0012$; Fig. 4*A*). However, this alteration in V_{max} was transient and disappeared after 48 h of co-incubation (0.21 ± 0.01 *vs.* 0.25 ± 0.01 lengths s^{-1}, $P = 0.099$; Fig. 4*A*). No significant differences in stress were observed between control and CD4^{+} T cell-exposed muscle strips at 24 h (84.5 ± 13.8 *vs.* 107.8 ± 4.8 kPa; control *vs.* T cells, $P = 0.1$) or 48 h (99.6 ± 5.7 *vs.* 92.0 ± 5.2 kPa, $P = 0.47$; Fig. 4*B*). Representative data from a control muscle strip and from one incubated with OVA-stimulated CD4^{+} T cells are presented in Fig. 4*C*.

Western blot analysis of contractile protein expression in ASM co-cultured with OVA-stimulated CD4^{+} T cells

After 24 h incubation with OVA-stimulated CD4^{+} T cells, significant increases in levels of both MLCK (0.2 ± 0.03 *vs.* 0.41 ± 0.09 a.u.; control *vs.* T cells, $P = 0.018$; Fig. 5*A*) and the (+)insert myosin isoform (0.55 ± 0.04 *vs.* 0.81 ± 0.05 a.u.; control *vs.* T cells, $P = 0.0023$; Fig. 5*B*) were observed. There was no difference in total SMMHC (0.69 ± 0.04 *vs.* 0.70 ± 0.07 a.u.; control *vs.* T cells, $P = 0.77$; Fig. 5*C*) or of α-smooth muscle actin (1.2 ± 0.06 *vs.* 1.3 ± 0.1 a.u., control *vs.* T cells, $P = 0.36$; Fig. 5*D*). The ratio of (+)insert to total SMMHC was, however, significantly higher in ASM co-cultured with
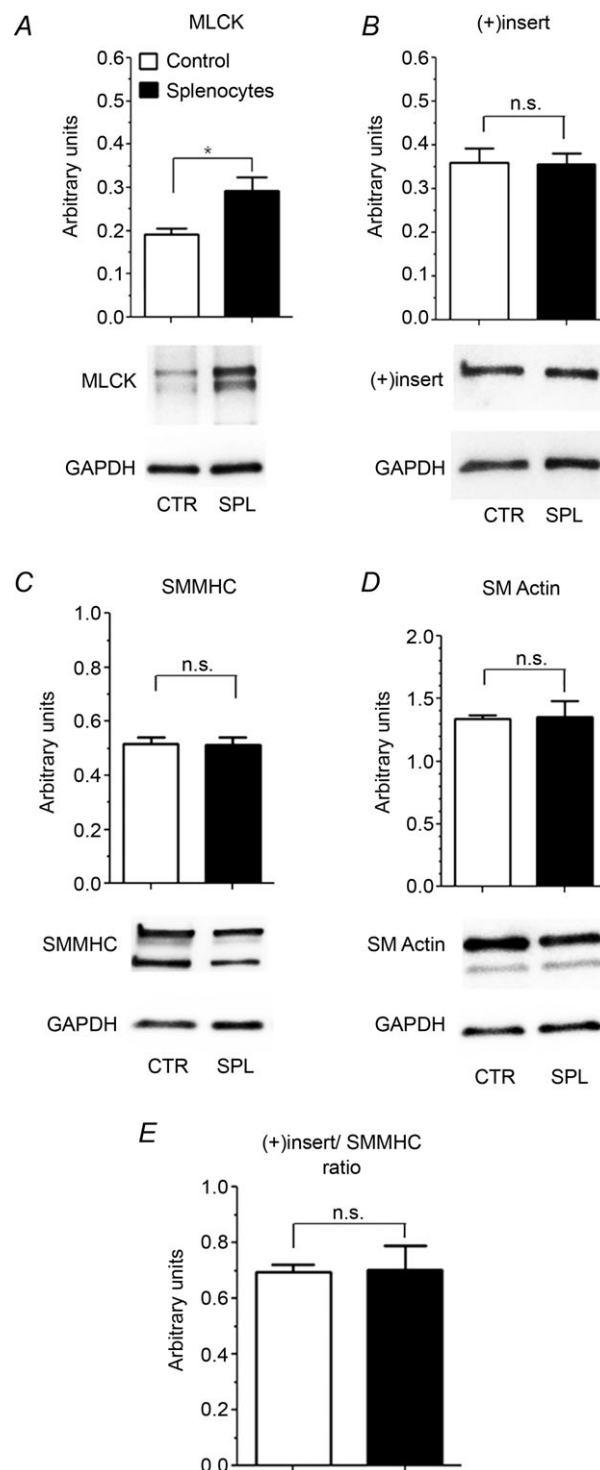
Figure 3. Western blot analysis of myosin light chain kinase (MLCK; *A*), 7-amino acid insert in N-terminal part of smooth muscle myosin [(+)insert SMMHC; *B*], total SMMHC isoform (*C*), α-smooth muscle actin (SM actin; *D*) and (+)insert to total SMMHC ratio (*E*) from ASM strips incubated in the absence (CTR; open columns) or in the presence of OVA-stimulated splenocytes (SPL; filled columns)
$n = 3$ rats per group. Representative Western blots are shown. *$P < 0.05$.
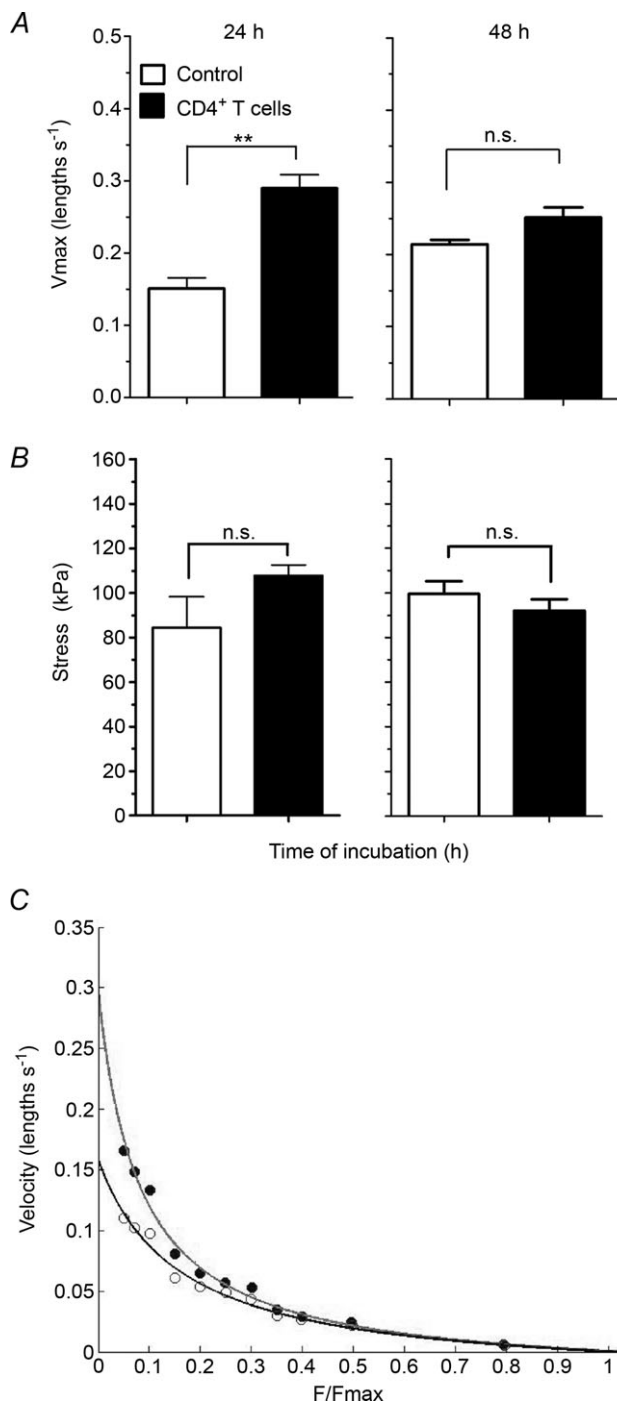
Figure 4. Mechanical properties of ASM incubated with OVA-stimulated CD4$^+$ T cells

A, V_{max} of ASM incubated in the absence (open columns) or presence of OVA-stimulated CD4$^+$ T cells (filled columns) for 24 or 48 h. B, stress generated by ASM incubated in the absence (open columns) or presence of OVA-stimulated CD4$^+$ T cells (filled columns) for 24 or 48 h. C, representative force–velocity curves of BN ASM co-incubated in the absence (open circles) or in the presence of CD4$^+$ T cells (filled circles) for 24 h. F/Fmax: Force normalized to the maximal Force. Force –velocity relationships were accurately fitted by the Hill hyperbolic model, $r^2 > 0.96$. $n = 5$ rats per group. *$P < 0.05$.

OVA-stimulated CD4$^+$ T cells (0.83 ± 0.11 vs. 1.18 ± 0.16; control vs. T cells, $P = 0.024$; Fig. 5E). These alterations in contractile protein levels were transient, because at 48 h, levels of MLCK (0.16 ± 0.02 vs. 0.13 ± 0.02 a.u.; control vs. T cells, $P = 0.43$; Fig. 5A) and (+)insert isoform (0.54 ± 0.003 vs. 0.51 ± 0.03 a.u.; control vs. T cells, $P = 0.42$; Fig. 5B) were not statistically different. Furthermore, no differences were observed at 48 h in total SMMHC (0.70 ± 0.07 vs. 0.66 ± 0.04 a.u.; control vs. T cells, $P = 0.72$; Fig. 5C), α-smooth muscle actin (1.34 ± 0.03 vs. 1.36 ± 0.05 a.u.; control vs. T cells, $P = 0.83$; Fig. 5D) and in the ratio of (+)insert to total SMMHC (0.79 ± 0.08 vs. 0.77 ± 0.05; control vs. T cells, $P = 0.7$; Fig. 5E).

Effect of OVA-stimulated CD4$^+$ T cell incubation on the phosphorylation level of LC$_{20}$

Knowing that the incubation of ASM with OVA-stimulated CD4$^+$ T cells for 24 h increased the expression levels of MLCK, we tested whether or not this led to a corresponding increase in myosin LC$_{20}$ phosphorylation. Indeed, the LC$_{20}$ phosphorylation level was significantly increased in muscle strips co-cultured with OVA-stimulated CD4$^+$ T cells compared with control conditions ($31.7\% \pm 4.1$ vs. $40.5\% \pm 2.4$; control vs. T cells, $P = 0.022$; Fig. 6).

Mechanics of ASM co-cultured with or without contact with OVA-stimulated CD4$^+$ T cells

To assess whether or not direct contact between OVA-stimulated CD4$^+$ T cells and the ASM was required to enhance muscle mechanics, CD4$^+$ T cells and ASM were co-incubated in a Transwell chamber, separated by a permeable membrane, that allows transfer of small molecules (such as cytokines) but not cells. For ASM that was in contact with co-cultured CD4$^+$ T cells, a significant increase in V_{max} was observed (0.31 ± 0.02 lengths s^{-1}) compared with that obtained when tracheal rings were co-cultured with CD4$^+$ T cells in the absence of contact (0.25 ± 0.01 lengths s^{-1}) or compared with tracheal rings incubated in medium alone (0.24 ± 0.01 lengths s^{-1}; $P = 0.01$; Fig. 7A). Again, stress did not change significantly between the ASM co-cultured with the OVA-stimulated CD4$^+$ T cells in direct contact (85.8 ± 7.7 kPa), without contact (95.2 ± 3.4 kPa) or incubated in medium alone (81.4 ± 7.2 kPa; $P = 0.33$; Fig. 7B).

Discussion

The major finding of this study is that incubation of BN rat tracheal rings with OVA-stimulated CD4$^+$ T cells enhanced the unloaded V_{max}. This increase in V_{max}
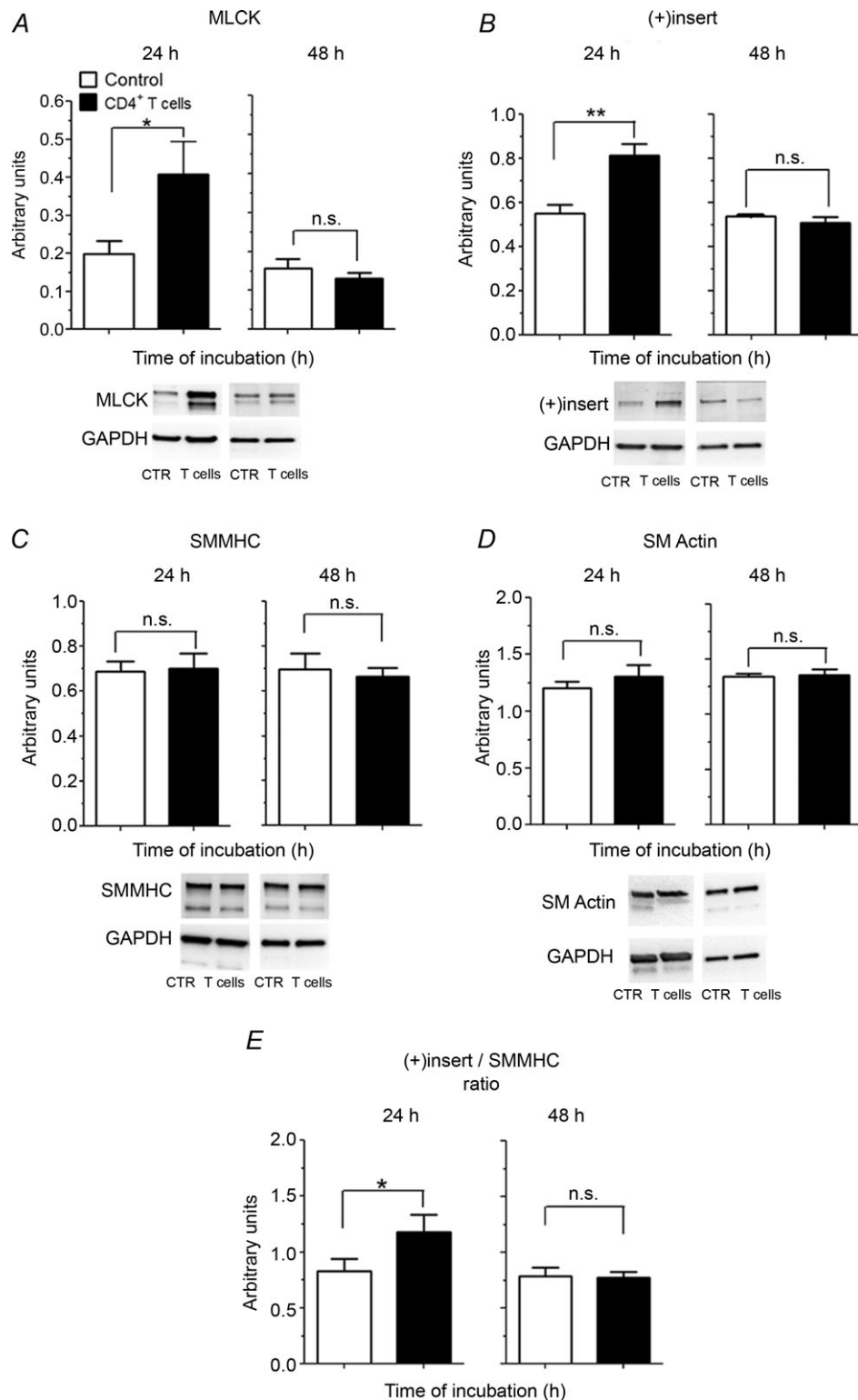
Figure 5. Western blot analysis of MLCK (*A*; *n* = 7 rats per group for 24 h and *n* = 3 rats per group for 48 h), (+)insert SMMHC isoform (*B*; *n* = 6 rats per group for 24 h and *n* = 3 rats per group for 48 h), SMMHC (*C*; *n* = 7 rats per group for 24 h and *n* = 3 rats per group for 48 h), α-smooth muscle actin (*D*; *n* = 6 rats per group for 24 h and *n* = 3 rats per group for 48 h) and ratio of (+)insert SMMHC to total SMMHC (*E*; *n* = 6 rats per group for 24 h and *n* = 3 rats per group for 48 h) for ASM incubated in the absence (open columns) or in the presence of OVA-stimulated CD4$^+$ T cells (filled columns)
Representative Western blots are shown. *$P < 0.05$; **$P < 0.01$.

was accompanied by increased levels of the (+)insert SMMHC isoform and MLCK. Furthermore, these changes in mechanics were dependent on contact between the $CD4^+$ T cells and the ASM cells.

As seen in the literature, it has been difficult to define the precise role of ASM in AHR and asthma. A number of studies have reported increases in V_{max} in ASM strips from animals with experimentally induced or innate AHR (Antonissen *et al.* 1979; Jiang *et al.* 1992*b*; Fan *et al.* 1997; Gil *et al.* 2006). An increase in V_{max} was also reported in human asthmatic ASM cells (Ma *et al.* 2002) and bronchial rings sensitized with IgE from asthmatic subjects (Mitchell *et al.* 1994), while others did not observe such differences (Chin *et al.* 2012). An increase in force-generating capacity has also been reported in mice (Chiba *et al.* 2009) and rabbit ASM bundles (Grunstein *et al.* 2002). However, the literature on human ASM force-generating capacity is controversial. The force normalized to mass generated

by asthmatic ASM strips from the second to the fourth airway generations has been reported to be greater than that of non-asthmatic ASM by Thomson *et al.* (1996), whereas Chin *et al.* (2012) did not show any difference in tracheal ASM stress. Taken together, these studies suggest that ASM might be hypercontractile in AHR and asthma, but which mechanical property or properties is/are altered and/or which airways are affected have remained elusive. Irrespective of these issues, the question addressed in the present study is: can inflammation, as encountered in asthma, lead to ASM hypercontractility and, if so, by which mechanism?
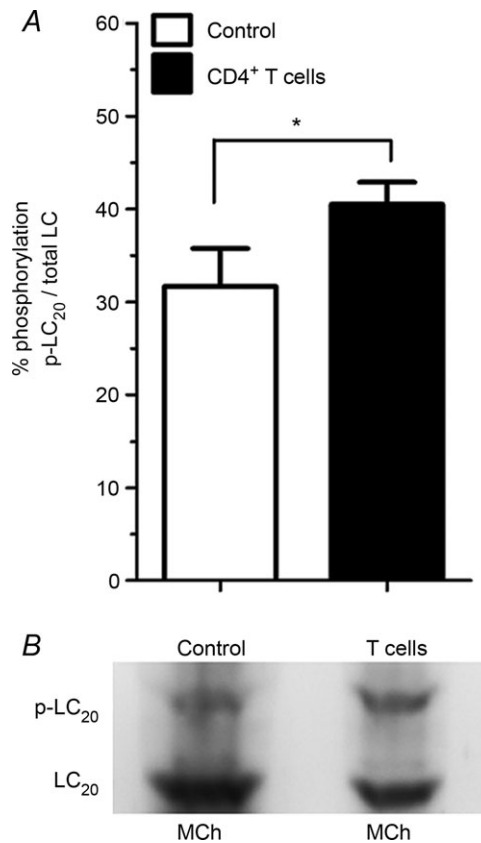

Figure 6. Phosphorylation of myosin LC_{20}
A, response to acetyl-*β*-methylcholine chloride (MCh) stimulation in ASM incubated for 24 h in the absence (open columns) or in the presence of OVA-stimulated $CD4^+$ T cells (filled columns). *B*, a representative Western blot of phos-tag gel of phosphorylated (p-LC_{20}) and total LC_{20} (LC_{20}) after MCh stimulation of a muscle strip incubated in the absence (control) or in the presence of OVA-stimulated $CD4^+$ T cells (T cells). *P < 0.05. *n* = 6 rats per group.
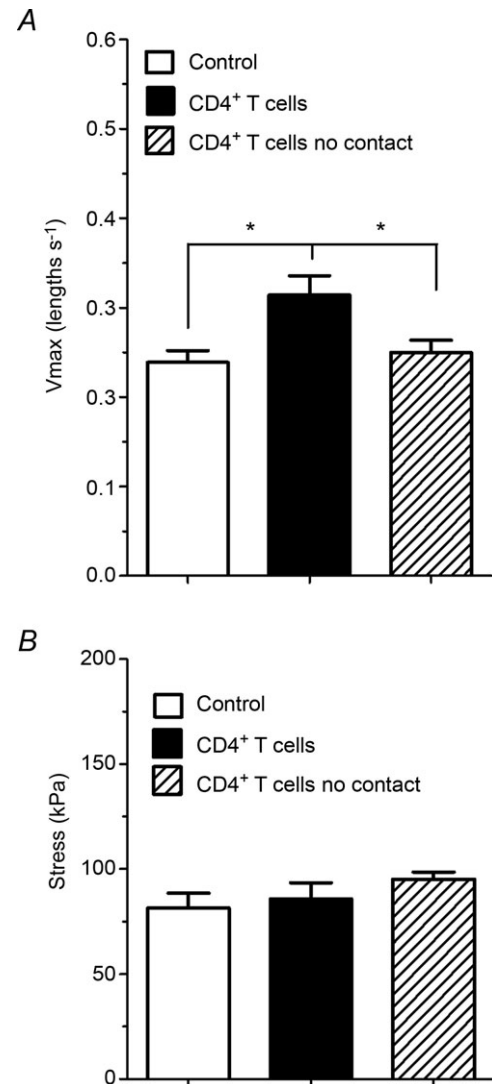

Figure 7. Mechanical effect of contact between OVA-stimulated $CD4^+$ T cells and ASM strips
The V_{max} (*A*) and stress (*B*) measured for ASM strips incubated for 24 h in the absence of $CD4^+$ T cells (open columns) or in the presence of $CD4^+$ T cells either in contact (filled columns) or in Transwells (hatched columns). *n* = 7 rats per group. *P < 0.05.

Twenty-four hours of incubation of tracheal rings with purified OVA-stimulated CD4$^+$ T cells led to significant increases in V_{max} (Fig. 4A) and in levels of MLCK (Fig. 5A) and the (+)insert SMMHC isoform (Fig. 5B). Myosin light chain kinase is the enzyme responsible for myosin activation by phosphorylation of myosin light chain, LC$_{20}$ (Small & Sobieszek, 1977), whereas myosin light chain phosphatase deactivates it (Sobieszek *et al.* 1997). Thus, LC$_{20}$ phosphorylation levels depend on the amount and activity of MLCK and myosin light chain phosphatase. The interaction between all these factors is complex (Jiang *et al.* 1992a, 1995). Nevertheless, in our rat tissues, exposure to OVA-stimulated CD4$^+$ T cells resulted in a significant increase in myosin LC$_{20}$ phosphorylation levels (Fig. 6). A direct relationship between V_{max} and LC$_{20}$ phosphorylation has, however, not been established (Moreland *et al.* 1986; Miller-Hance & Kamm, 1991; Gunst *et al.* 1994), but an increase in force (or stress) would have been expected (Silver & Stull, 1982). While there was a trend towards an increase in stress, it did not reach statistical significance (Fig. 4B). Importantly, the ragweed-sensitized canine model of AHR (Becker *et al.* 1989) also showed increases in MLCK levels, but not in activity (Jiang *et al.* 1992b), and increases in LC$_{20}$ phosphorylation levels and in V_{max}, but no changes in stress (Jiang *et al.* 1992a, 1995). Thus, our *in vitro* experiments of muscle stimulation by inflammatory cells produced data consistent with this whole-animal model of allergen sensitization (Becker *et al.* 1989).

The expression of MLCK in asthma has been studied extensively. Some studies showed that its expression was not altered in asthma (Woodruff 2008; Matsumoto *et al.* 2007), whereas others showed an increase at the mRNA level (Ma *et al.* 2002; Léguillette *et al.* 2009) and at the protein level using immunoblotting (Ammit *et al.* 2000) and immunohistochemistry (Benayoun *et al.* 2003). Benayoun *et al.* (2003) even reported a positive correlation between MLCK content and asthma severity, whereas the expression pattern of α-actin and total SMMHC was similar in asthmatic and non-asthmatic subjects. We also report a similar content in α-actin and total SMMHC between OVA-activated, T-cell-exposed and control ASM strips (Fig. 5C and D), suggesting that there is no change in muscle mass. The fact that there is no change in total myosin normalized to the total protein loaded (Fig. 5C) may contribute to the fact that we did not observe a significant increase in stress (Fig. 4B).

Incubation of the ASM strips with the OVA-activated CD4$^+$ T cells also led to an increase in the (+)insert SMMHC isoform. In mammalian smooth muscle, four SMMHC isoforms are generated by alternative mRNA splicing. Two of these isoforms (White *et al.* 1993) differ in the N-terminus by the presence or absence of a 7-amino acid insert near the ATPase site. They are referred to as the (+)insert or SMB and the (−)insert or SMA isoforms,

respectively. The (+)insert isoform is preferentially expressed in phasic, rapidly contracting smooth muscle, whereas the (−)insert isoform is found predominantly in tonic, slowly contracting smooth muscle (Kelley *et al.* 1993). Both isoforms are found in ASM, but the expression of the (+)insert isoform has been shown to be greater in the trachea of an animal model of innate AHR (mRNA and protein levels; Gil *et al.* 2006) as well as in asthmatic end-obronchial biopsies (mRNA level studied only; Leguillette *et al.* 2009). The (+)insert SMMHC isoform has a 2-fold greater ATPase rate (Kelley & Adelstein, 1994) and velocity of propulsion of actin in *in vitro* motility assays compared with the (−)insert isoform (Rovner *et al.* 1997; Lauzon *et al.* 1998). Thus, the (+)insert SMMHC isoform leads to a greater velocity of shortening. Its overexpression after exposure of the rat trachealis to the CD4$^+$ T cells is likely to play a major role in the increase in V_{max} measured.

Incubation of the ASM strips with the CD4$^+$ T cells led to an increase in MLCK (Fig. 5A), (+)insert SMMHC isoform (Fig. 5B) and V_{max} (Fig. 4A), whereas incubation with total splenocytes increased only MLCK levels (Fig. 3A) and did not alter the mechanics (Fig. 2). The CD4$^+$ T cells comprise ∼25% of the total splenocyte population after 4 days of incubation with OVA and thus, it is possible that total splenocytes did not contain a large enough number of CD4$^+$ T cells to alter the contractile protein expression significantly and thus, increase V_{max}. Alternatively, the presence of other cells, such as CD8$^+$ cells or B cells, might have suppressed the effect of the CD4$^+$ T cells (Mishima *et al.* 1998).

In order to understand better the mechanism by which CD4$^+$ T cells altered ASM contractility, we examined whether the observed effects required contact between the ASM and the CD4$^+$ T cells or simply involved the release of mediators. Our results (Fig. 7) clearly demonstrate that contact is necessary to alter V_{max} and suggest that CD4$^+$ T cell cytokines alone are not sufficient in the absence of direct contact. These interactions between T cells and ASM may be mediated by the intercellular cell adhesion molecule 1, vascular cell adhesion molecule 1 or CD40 and OX40 ligand, all of which are expressed by ASM cells, and by CD44, lymphocyte function-associated antigen 1 and very late antigen 4 expressed by the T cells (Lazaar *et al.* 1994; Burgess *et al.* 2005; Al Heialy *et al.* 2013). Further studies will be required to examine the contribution of these (and potentially other) molecules to the enhanced contractile responses we have observed.

The changes in mechanics (increased V_{max}) observed after 24 h of incubation with the CD4$^+$ T cells were only transient (Fig. 4); differences in V_{max} between control and the CD4$^+$ T cells were no longer present after 48 h of co-incubation (Fig. 4A). Again, these results were correlated with the changes in levels of the contractile proteins; that is, both MLCK (Fig. 5A) and the (+)insert SMMHC isoform (Fig. 5B) had returned to control levels

after 48 h of exposure to the CD4$^+$ T cells. Several factors may have contributed to the transient aspect of the responses of our muscle strips to the CD4$^+$ T cells. Firstly, models of allergic asthma usually require multiple allergen exposures (Becker *et al.* 1989). Our muscle strips were exposed only once to the CD4$^+$ T cells because of technical limitations of *in vitro* tissue studies; that is, in preliminary experiments we cultured ASM strips for longer periods of time and observed changes in mechanics after 72 h of incubation even in the absence of inflammatory cells. The most likely explanation for these alterations is the presence of DMEM containing 0.5% FBS, a necessary component of the culture medium for the survival of the tissues (Hsieh & Farley, 2002). Indeed, Hsieh & Farley (2002) showed that swine tracheal muscle strips cultured in DMEM with serum concentrations from 1 to 10% exhibited a decreased M$_2$ muscarinic expression after 48 h of culture. Given that the M$_2$ receptors limit the release of acetylcholine, a decrease in their expression due to the presence of FBS leads to increases in ASM contractility (Fryer *et al.* 1999). Our FBS concentration was lower (0.5%), so this effect may have taken longer to come into play. Thus, to limit the artifacts due to tissue culture *per se*, we performed our measurements over a short period of time and so did not address the effect of repeated stimulation. Another possible reason that would explain why our responses were transient is the fact that we studied the CD4$^+$ T cells in isolation from other inflammatory cells. The CD4$^+$ T-cell-induced AHR is associated with eosinophilia in the BN rat (Mishima *et al.* 1998). A direct interaction between activated human CD4$^+$ T cells and eosinophils occurs through the intercellular cell adhesion molecule 3 receptor (Douglas *et al.* 2000). Moreover, cytokines released by human CD4$^+$ T cells regulate the priming, activation and survival of eosinophils (Rothenberg *et al.* 1988). Thus, interactions between these two cell types might be necessary for long-term effects. In addition, Th2-cytokines (interleukin-4 and interleukin-5) are known to prime human mast cells (Ochi *et al.* 2000), and the mast cells can interact with ASM through CD44 and CD51 receptors (Girodet *et al.* 2010). Thus, a whole array of interactions between inflammatory cells is likely to be necessary to orchestrate the ASM mechanical alterations and their maintenance.

Contrary to what might have been expected, the mechanical parameter that was readily affected by the CD4$^+$ T cells was V_{max} and not stress. As mentioned above, this was also the case in the canine ragweed allergic model of AHR (Jiang *et al.* 1992*a*, 1995). Intuitively, one might expect that asthmatic airways would have stronger ASM, thus leading to a greater bronchoconstriction upon simulation. This has not been demonstrated convincingly in asthmatic tissues and remains a topic under debate (Thomson *et al.* 1996; Chin *et al.* 2012). Nonetheless, it is conceivable that faster ASM would also lead to

greater airway resistance by counteracting the relaxing effect of tidal breathing (Gunst, 1983; Solway & Fredberg, 1997). Indeed, ASM stretching by tidal or deep breaths decreases airway resistance in normal lungs but not in asthmatic patients (Fish *et al.* 1981; Skloot *et al.* 1995). Also, imaging techniques have demonstrated that the airways of asthmatic patients dilate upon stretching but that this is brief because they quickly narrow back to their initial diameter (Brown *et al.* 2001). This suggests that asthmatic ASM can shorten faster than normal ASM after a stretch. This rapid recontraction might maintain asthmatic airways in a more constricted state on average because the ASM has time to shorten significantly between each breath, thus counteracting the relaxing effect of tidal breaths. Despite this, a number of animal model studies have reported that incubation of ASM with Th2 cytokines enhances their isometric force and impairs their relaxation (Hakonarson *et al.* 1999; Grunstein *et al.* 2002; Whelan *et al.* 2004; Chiba *et al.* 2009). None of those studies addressed the velocity of shortening. As noted above, the stress that we reported in Fig. 4*B* showed only a tendency to increase after incubation with the CD4$^+$ T cells but did not reach statistical significance. It is likely that the concentrations of cytokines used in the other studies were higher than those obtained from the CD4$^+$ T cells used in our experiments. Furthermore, we report stress (force per cross-sectional area; Chin *et al.* 2010), whereas these other studies reported force per gram of muscle, which can be erroneously affected by muscle length (Mehta *et al.* 1996).

In conclusion, our data demonstrate that contact between CD4$^+$ T cells and ASM enhances the mechanics of ASM, but in a transient manner. More work will be required to delineate whether repeated challenges and/or interactions between several inflammatory cell candidates and their cytokines can lead to more permanent alterations in ASM mechanical properties, as observed in asthma.

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Additional information

Competing interests

None declared.

Author contributions

O.S.M., E.M.N. and L.K. performed the experiments; O.S.M., E.D.F. and A.-M.L. wrote the manuscript; all co-authors were involved in the design of the experiments, the analysis and interpretation of the data and approval of the final version of the manuscript.

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